



PATENT
Attorney Docket No. DHI-03864

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: James L. Brown

Serial No.: 09/539,735

Group No.: P. Nolan

Filed: 03/30/00

Examiner: 1644

Entitled: DIAGNOSIS OF AUTOIMMUNE DISEASE

**DECLARATION UNDER 37 C.F.R. § 1.132
BY DR. LEONARD KOHN**

Commissioner for Patents

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CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: 5-12-04

By: Clara

Sir:

1. I, Leonard Kohn, am the subject of the Curriculum Vitae that was previously mailed to the Office in this application on December 2, 2002, and author of the publications shown on the list attached thereto. On the basis of the information and facts contained in those documents, I submit that I am qualified to speak on the level of ordinary skill in the art of the claimed invention.

2. The Examiner rejected Claims 1, 3-16 and 18-33 for alleged obviousness over Evans *et al.*¹ in view of Yamashiro *et al.*,² arguing that the levels of cAMP generation were "directly correlated" to the level of gene expression detection by a luminometer.

¹ Evans *et al.* (1999) "Development of a luminescent bioassay for thyroid stimulating antibodies," *J. Clin. Endocrin. Metabolism* 84(1)374-377.

² Yamashiro *et al.* (1999) "Mechanism of the Augmentative Effect of High Polyethylene Glycol (PEG) Concentrations on the Thyroid Stimulating Activity in TSAb-IgG Using a Porcine Thyroid Cell Assay," *Endocrine Research* 25:67-75.

3. **The data shows that reporter gene expression in CHO-Rluc cells is not mediated by cAMP signaling, but rather is mediated by arachidonate/Ca/IP signaling**

A. It is known in the art that several signaling pathways are involved in expression of Graves's disease, including the cAMP signal and the arachidonate/Ca/IP signal.³ The cAMP signal is mediated by phosphokinase A (PKA)⁴ and requires insulin/IGF-1 and the PI3 kinase.⁵ In contrast, the arachidonate/Ca/IP signal is mediated by phosphokinase C (PKC).⁶

B. To test which of these signals mediates reporter gene expression in CHO-Rluc cells, Dr. Giorgio Napolitano at the University G.G. "Enuncio" of Chieti, Italy, evaluated the action of inhibitors of the PKA, IP3 kinase, and PKC pathways on gene expression in these cells. Briefly, luciferase gene luminescence was determined following incubation of CHO-Rluc cells with thyroid stimulating hormone (TSH), randomly selected Graves' sera from 5 patients which were previously determined to increase cAMP release in a cAMP immunoassay, a commercially available (Diagnostic Hybrids, Inc., Ohio) positive control of sera from a pool of Graves' patients with increased cAMP values (referred to as "C+"), and a commercially available (Diagnostic Hybrids, Inc., Ohio) negative control of pooled normal sera (referred to as "C-"). These incubations were done in the presence or absence of 10 µM H89 (an inhibitor of PKA),⁷

³ Di Cerbo *et al.*, Signaling pathways involved in thyroid hyperfunction and growth in Graves' disease. Biochimie. 1999; 81: 415-24; Di Cerbo, *et al.* Graves' immunoglobulins activate phospholipase A2 by recognizing specific epitopes on the TSH receptor. J. Clin. Endocrinol. Metab. 1999; 84: 3283-3292.

⁴ Tasken K, Aandahl EM. Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol Rev, 84, 137-167 (2004).

⁵ Saji M, Kohn LD. Insulin and insulin-like growth factor-I inhibit thyrotropin-increased iodide transport in serum-depleted FRTL-5 rat thyroid cells: modulation of adenosine 3',5'-monophosphate signal action. Endocrinology, 128, 1136-1143 (1991), Kimura T, Van Keymeulen A, et al Regulation of thyroid cell proliferation by TSH and other factors : acritical evaluation of in vitro models. Endocr Rev 22, 631-656 (2001).

⁶ Khan WA, Blobe GC, Hannun YA. Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C. Cell Signal, 7, 171-184 (1995).

⁷ Leemhuis J *et al.* The protein kinase A inhibitor H89 acts on cell morphology by inhibiting Rho kinase. J Pharmacol Exp Ther 300, 1000-1007 (2002), Davies SP *et al.* Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351, 95-105, (2000).

500 μ M Wortmannin (inhibitor of PKC and PI3kinase),⁸ or 20 μ M LY294002 (inhibitor of PI3kinase).⁹ Control samples did not receive inhibitor. The data is shown in Table 1.

Table 1
Level of luciferase gene expression as determined by luminescence in
CHO-Rluc cells in the presence of PKA inhibitor (H89),
PKC inhibitor (Wortmannin), or PI3 kinase inhibitors (Wortmannin and LY294002)^(a)

Serum No.	BLANK AVERAGE	H 89 AVERAGE	WORT AVERAGE	LY294002 AVERAGE	ETYA AVERAGE
BLANK	606.5	986	<u>143.5</u>	3100.5	987
TSH	15442	22134.5	34353.5	26951.5	17211.5
2341	2609	6726.5	<u>171.5</u>	12248	2498.5
2586	2275.5	6604	<u>217.5</u>	15665	2518.5
2850	1342	5552	<u>218</u>	9737	2231
5046	4877	10826	9407.5	10828.5	6266
3515	20029.5	26593	30009.5	23762	-
C+	9341	12339	17458.5	16851.5	7640
C-	2173	2693.5	4040.5	6436	1906
NIS Gene	23,050	15,410	-	-	-

^(a) All samples were in duplicate. Values are the average of multiple experiments. Increased and decreased values are significant at P<0.05 in Student t test. Bold values are significantly increased compared to control samples, bold and underlined values are significantly decreased compared to control samples.

Table 1, 3rd column, shows that H89, the PKA inhibitor, did **not inhibit** reporter gene expression in the assay of 5 individual Graves' patients' sera, the commercially available pooled positive control serum (C+), or even TSH. Rather there was an **increase** in reporter gene expression when PKA (which mediates the cAMP signal) was inhibited by H89! Importantly, this result was not due to a lack of H89 activity, since Table 1 shows 40% inhibition of sodium

⁸ Cross MJ *et al.* Wortmannin and its structural analogue demethoxyviridin inhibit stimulated phospholipase A2 activity in Swiss 3T3 cells. Wortmannin is not a specific inhibitor of phosphatidylinositol 3-kinase. J Biol Chem 270, 25352-25355, (1995).

⁹ Vlahos CJ, Matter WF, Hui KY, Brown RA. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem, 269, 5241-5248 (1994).

iodide symporter (NIS) gene expression by H89, which is consistent with the inhibition previously described for this gene.¹⁰

Thus, the absence of H89 inhibition of PKA activity shown Table 1 demonstrates that reporter gene expression in CHO-Rluc cells is **not** mediated by increased levels of released cAMP. This is contrary to the Examiner's assertion that the released levels of cAMP are "directly correlated" to the levels of reporter gene expression in CHO-Rluc cells. In view of this, and taken one step further, one of ordinary skill in the art would not reasonably expect that PEG, even if it arguably increased cAMP levels, would also increase reporter gene expression in CHO-Rluc cells.

C. In stark contrast to the increased gene expression in the presence of H89 (3rd column of Table 1), Wortmannin inhibited reporter gene expression in 4 of the 6 individual sera tested (4th column of Table 1), whereas LY294002 increased gene expression in each of these sera (5th column of Table 1). Unlike LY294002, which is a specific PI3kinase inhibitor, Wortmannin inhibits PI3kinase mediated AKT activity as well as PKC mediated activity. Thus, Table 1 shows that reporter gene expression in CHO-Rluc cells is **not** mediated in any of the tested Graves' patients' sera via a cAMP signal, but rather is mediated in 67% of these patients via the PKC signal and the arachidonate/Ca/IP signal.

D. The above-discussed data and conclusions in item 3.B. is consistent with the observations of Yamashiro *et al.* (the secondary reference cited by the Examiner). In particular, Yamashiro *et al.* expressly noted that cAMP release was not stimulated by "thyroid stimulators such as TSH, forskolin, GTP γ S and pituitary adenylate cyclase activating polypeptide (PCAP)."¹¹ This observation was also confirmed by Innui *et al.*¹² who found that "no stimulatory action by PEG was found with the thyroid stimulating substances such as GTP γ S, forskolin or pituitary adenylate-cyclase activating polypeptide (PACAP)."¹³ Thus, one of ordinary skill in the art reading Yamashiro *et al.* and Innui *et al.* would expect that the action of thyroid stimulators (including PEG which is recited in the claims) is **not** necessarily mediated by

¹⁰ Garcia B, Santisteban P. PI3K is involved in the IGF-I inhibition of TSH-induced sodium/iodide symporter gene expression. Mol Endocrinol, 16: 342-352 (2002).

¹¹ Yamashiro *et al.*, page 71, last paragraph.

¹² Innui *et al.* (1998) "Increase of Thyroid Stimulating Activity in Graves' Immunoglobulin-G by High Polyethylene Glycol Concentrations Using Porcine Thyroid Cell Assay," Thyroid 8:319-325.

¹³ Innui *et al.*, Abstract, and Figure 8.

cAMP release. This expectation is further borne out by the data in Table 1, which further shows that the action of thyroid stimulators is mediated via an **alternative pathway** of arachidonate/Ca/IP signal.

4. **Evans et al.'s alleged correlation is the result of sample selection, and there is a low degree of correlation between cAMP and gene expression in CHO-Rluc cells when using these selected samples**

A. In addition, the apparent “correlation” between released cAMP levels and luciferase expression in Evans *et al.*’s Figure 3 is the result of the authors’ selection of particular samples for representation in that Figure. In particular, Evans *et al.* divided their patients according to the presence TBII levels as shown in Figure 2, with 50 “TBII negative” and 50 TBII positive samples. Figure 2, shows that there a correlation between increased luciferase gene expression in TBII positive samples (compare luminescence between GD+ and GD- samples in Figure 2).

Importantly, Evans *et al.*’s Figure 3 (which formed the basis of the Examiner’s conclusion of a “direct correlation” between increased cAMP levels and gene expression levels), shows the relationship between luciferase expression and released cAMP levels using only 44 of the 100 sera, 35 of which are TBII positive.¹⁴ Because Evans *et al.* selected TBII positive samples, and because TBII were known in the art to increase arachidonate/Ca/IP signaling,¹⁵ one of ordinary skill in the art would reasonably conclude that the increased gene expression observed in the predominantly TBII positive samples of Figure 3 is mediated by an increase in the arachidonate/Ca/IP signal rather than by an increase in cAMP signal. This further demonstrates that one of ordinary skill in the art would **not** have had a reasonable expectation that increased gene expression in CHO-Rluc cells in Evans *et al.* correlated with released cAMP levels and

¹⁴ Evans *et al.*, Figure 3 legend states that the results show “[c]orrelation ... in 44 treated GD sera (35 TBII positive).”

¹⁵ Marcocci, *et al.* Norepinephrine and thyrotropin stimulation of iodide efflux in FRTL-5 thyroid cells involves metabolites of arachidonic acid and is associated with the iodination of thyroglobulin. *Endocrinology* 120, 1127-1133 (1987); Kohn, *et al.* Characterization of monoclonal thyroid stimulating and thyrotropin binding inhibiting autoantibodies from a Hashimoto’s patient whose children had intrauterine and neonatal thyroid disease. *J. Clin. Endocrinol. Metab.*, 82, 3998-4009 (1997); Sarlis, *et al.* Graves’ disease following thyrotoxic painless thyroiditis in two cases. Analysis of antibody activities against the thyrotropin receptor (TSHR) and etiopathogenic implications. *Thyroid*, 7, 829-836 (1997); Wortsman, *et al.* Thyrotropin receptor epitopes recognized by Graves’ autoantibodies developing under immunosuppressive therapy. *J. Clin. Endocrinol. Metab.*, 83, 2302-2308 (1998).

B. Even among the selected samples, there was a low degree of correlation between the level of released cAMP and gene expression in CHO-Rluc cells. For Example, using 44 samples of Graves' disease sera, Evans *et al.* states that,

“6 were positive by RIA [of cAMP] but negative in the luminescence assay as shown in figure 3.”¹⁶

This statement clearly shows that 6 of the 44 samples gave **diametrically opposite** results when assayed based on gene expression and released cAMP levels, respectively. Therefore, one of ordinary skill in the art, including myself, would conclude from this statement that gene expression and cAMP levels are **not** directly correlated in Evans *et al.*'s CHO-Rluc cells. Moreover, based on this, one of ordinary skill in the art, including myself, would **not** reasonably predict that gene expression will be elevated by PEG even if released cAMP levels were elevated by PEG.

C. Furthermore, while Evans *et al.*'s Figure 3 refers to “correlation” of TSAb as measured by gene expression (as determined by luminescence) versus released cAMP levels, a closer examination of Figure 3 reveals that the degree of correlation was extremely low with $r=0.6$, as expressly stated by Evans *et al.* in the legend to Figure 3. Indeed, fully 14 of the 44 samples¹⁷ of Graves disease sera showed **no correlation** between cAMP levels and the levels of gene expression as measured by luminescence. This squarely contradicts the Examiner's allegation of a “direct correlation” between released cAMP levels and gene expression in Evans *et al.*'s CHO-Rluc cells.

D. Further still, even in those samples that were labeled as allegedly showing a “correlation,”¹⁸ the level of gene expression nonetheless showed considerable variance at a given cAMP level. For example, Figure 3 shows a **3-fold and 2-fold discrepancy** in luminescence at cAMP RIA of 6 and 2, respectively. This further contradicts a “direct correlation” between released cAMP levels and gene expression in Evans *et al.*'s CHO-Rluc cells.

¹⁶ Evans *et al.*, sentence bridging pages 375 and 376.

¹⁷ The number of samples showing no correlation was manually counted in the top left quadrant and lower right quadrant of Evans *et al.*'s Figure 3.

¹⁸ The samples showing alleged correlation appear in the top right quadrant and bottom left quadrant of Evans *et al.*'s Figure 3.

E. Although Evans *et al.* stated that phosphodiesterase inhibitors (which inhibit breakdown of cAMP) increased luminescence to the level of saturation,¹⁹ this statement has little probative value on inferring a correlation between released cAMP levels and gene expression because it is unsupported by data, as admitted by Evans *et al.* For example, there is no data showing whether gene expression and/or cAMP is dose responsive to the phosphodiesterase inhibitors.

F. Based on the above, on facts and references discussed in my earlier Declaration that was mailed to the Office on December 2, 2002, as well as on my 44 years of expertise in the relevant art, I aver that the success of the instantly claimed methods was not obvious or predictable to me, nor could have been obvious or predictable to one of ordinary skill in the art, over Evans *et al.* in combination with Yamashiro *et al.* More particularly, in view of the above, it is my opinion that the combination of Evans *et al.*'s selection of a majority of TBII positive samples, together with the low degree of correlation between the level of released cAMP and the level of gene expression in Evans *et al.*'s CHO-Rluc cells in those samples, does not provide one of ordinary skill in the art with a reasonable expectation that increasing cAMP by using PEG would also result in increasing gene expression by using PEG.

5. **A long-felt and unsatisfied need existed in the art for a more accurate assay for detecting thyroid stimulating antibodies**

Graves' Disease is an autoimmune disorder in which excessive amounts of thyroid gland hormones are produced and secreted in response to the autoantibodies, taking over control from TSH. Graves' Disease is one of the most common autoimmune diseases with a prevalence of greater than 1% in the US population. Because stimulating autoantibodies to the TSH receptors in the Graves' patient's serum is pathognomonic of the disease, the art has recognized since 1958, *i.e.*, for 42 years before the claimed invention, the importance of early detection of the stimulating autoantibodies to the TSH receptors in the Graves' patients' serum.

Although several assays for detecting the presence thyroid stimulating antibodies were developed during the 42 years prior to the advent of the instantly claimed invention, none of these assays has satisfied this need.

For example, following the discovery of the Long Acting Thyroid Stimulator (LATS) in the serum of Graves' patients in 1956,²⁰ a mouse bioassay was developed for its measurement in

¹⁹ Evans *et al.*, page 377, column 1, first paragraph.

²⁰ Adams, D.D. & Purves, H.D. (1956). Proc. U.of Otago Med. Sch., 34:11-12.

1958.²¹ This assay used mice that had previously received radioactive iodine which was taken up by their thyroids and converted into thyroid hormones. The assay consisted of injecting the treated mouse with purified immunoglobulins from a patient suspected of having Graves' and examining the mouse serum for radioactive thyroid hormone. However, this assays suffered from several shortcomings. For example, it required 1-2 weeks of preparation time for the mice, it required 4 to 8 mice per IgG sample, it required 1 day of assay time, it was positive in only 40-50% of the Graves' patients, and its activity did not correlate with disease severity.

In 1964,²² it was definitively proven that LATS is an immunoglobulin to the TSH receptors on the thyroid. Because of the shortcomings of the mouse bioassay, assays using slices of human thyroid tissue were used. The basis for these assays was that the Graves' IgG reacts with the TSH receptor on the thyroid cells, stimulating the production of cAMP which can be measured using a radioimmunoassay. However, these assays had several drawbacks since human thyroid tissue was not readily available, there was substantial variability in assay results, and the cells were not continuously culturable.

Assays using different cell types were used, including FRTL5 cells from rat thyroid.²³ FRTL5 cells became the standard for detecting Graves' IgG since they could be frozen and thawed when ready to use, could be frozen and cultured for many generations, and were responsive to human Graves' IgG by producing cAMP which could be measured by radioimmunoassay (RIA). However, the FRTL cell assay suffered from several drawbacks, including that it required 10-14 days incubation before the cells can be used in the assay, the TSH receptors are not human, the patient IgG must be isolated from serum and purified before use in the assay in order to provide maximum sensitivity, an immunoassay that takes about a day to perform is required to measure the cAMP produced by the FRTL cells, and the assay misses 10-15% of clinically diagnosed Graves' disease patients.

Alternatives to the FRTL-5 cell assay included porcine thyrocytes²⁴ because of their greater availability than human cells. While these cells avoided the incubation drawbacks of FRTL-5 cells, they still had major problems of tissue preparation, uniformity of preparation, and sensitivity, *i.e.*, some of the same shortcomings seen with human cell cultures. In addition, they were positive in only 60-70% of Graves' patients using cAMP radioimmunoassays.

²¹ McKenzie, J.M. (1952). Endocrinol., 63:865-868.

²² Kriss, J.P., *et al.* (1964). J. Clin. Endo. and Medtab., 24:1005-1028.

²³ US Patent No. 4,608,341 issued to Ambesi-Impiombato in 1986, and US Patent No. 4,609,622 issued to Kohn *et al.* in 1986.

²⁴ Innui, T. *et al.* (1998) Thyroid, 8:319-325.

The next generation of assays was the use of continuous cell lines (Chinese Hamster Ovary cells) that are stably transfected with the human TSH receptor.²⁵ These cells were improvements in that they did not require long incubation times with fortified medium or “starvation” periods but only a period of 4-5 days culture before use, and they contained human TSH receptors. However, they still required purified IgG from the patients’ sera, cAMP measurement was still by immunoassay and they failed to detect 15-20% of clinically diagnosed Graves’ disease patients.

An advance in the cAMP immunoassay procedure was the use of PEG to increase the frequency of positives in porcine cell assays measuring cAMP release in low salt medium.²⁶ Although serum could be used in the assay, the immunoassay remained time consuming. Importantly, cAMP release did not translate to signaling because PEG did not alter forskolin, gammaGTP, or CPAP induced cAMP release despite their ability to increase cAMP synthesis. Rather, the assay best correlated with TBII positive sera and increased TBII activity, which is a measure of binding and inhibition of signaling. Therefore, this assay did not accurately detect clinically diagnosed Graves’ disease patients.

The next advance in the assay involved using CHO cells that contain human TSH receptors and genetically engineering them to contain expression constructs in which the luciferase gene is driven by a cAMP responsive promoter, transiently expressed with the receptor. Thus, Graves’ IgG can be detected using the substrate luciferin for the luciferase enzyme and measuring light output directly from the cells. One such published assay by Evans *et al.* (1999)²⁷ presented data comparing the RIA method in CHO cells with human TSH receptors to the luciferase method (discussed above). However, the correlation between these two methods was only 60% when used to test 44 treated Graves’ sera.²⁸

Importantly, Morganthaler, who is a coauthor of Evans *et al.* (1999) and who is currently the Research Director of BRAHMS that sells TBII kits, went on to pursue binding assays rather than Evans *et al.*’s function assays for detecting thyroid stimulating antibodies based on his recent view that “Unfortunately, the use of bioassays is limited ...”²⁹

²⁵ Persani, L. *et al.* (1993) J. Endocr. Invest., 16:511-519.

²⁶ Innui, T. *et al.* (1998), Thyroid, 8:319-325.

²⁷ Evans *et al.* (1999) "Development of a luminescent bioassay for thyroid stimulating antibodies," J. Clin. Endocrin. Metabolism 84(1)374-377.

²⁸ Correlation was at r=0.6, p<0.001. Evans *et al.*, page 376, legend to Figure 3.

²⁹ Minich *et al.* (2004) JCEM 89:352-356.

Thus, despite the above advances, and until shortly before the advent of the instantly claimed invention, there remained a need for an assay that more accurately correlated the detection of thyroid stimulating antibodies with clinically diagnosed Graves' disease. This need was captured by Davies,³⁰ who stated:

"Many physicians would pay dearly to obtain a marker for ... [Grave's] disease that interests them most. ...The major hurdle remains one of increasing the sensitivity of the available assays for TSHR-Ab so that their usefulness can be applied successfully to an even greater proportion of patients with Graves' disease."

6. The long-felt need was satisfied by the instantly claimed methods

A. The results of experiments that were conducted by me or under my supervision showed that the instantly claimed methods reached a result that is superior to prior methods by substantially increasing the accuracy of detection of thyroid stimulating antibodies when testing serum from 45 patients that were clinically diagnosed with Graves' disease.

B. Based on my 44 years of experience in the relevant art and the above discussed data, it is my opinion that one of ordinary skill in the art would not have had a reasonable expectation of success in practicing the claimed methods based on the combined teachings of Evans *et al.* and Yamashiro *et al.*

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Dated: 5/12/04

By: Leonard D Kohn
Dr. Leonard Kohn

³⁰

T. F. Davies, in Thyroid-Stimulating Antibodies Predict Hyperthyroidism. JCEM 83:3777-3781 (1998).